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Spectrophotometric Tool for the Determination of the Total Carboxylate Content in Proteins; Molar Extinction Coefficient of the Enol Ester from Woodward's Reagent K Reacted with Protein Carboxylates

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A number of relevant properties of Woodward's reagent K have been determined, such as the stability of the reactant and the optimal reaction conditions of the reactant with protein carboxylates. A Woodward's reagent K stock solution was stable at 4 °C for prolonged time, whereas upon storage at 22 °C, almost 20% of the reactive compound was lost within 1 week. The pH-dependency of the spontaneous degradation reaction of Woodward's reagent K was studied and was shown to be base-mediated. A molar extinction coefficient of 3150 M^{-1} cm^{-1} at 269 nm for the enol ester resulting from the reaction between Woodward's reagent K and the protein carboxylates was established using the conditions laid out in this work. This value was validated using a variety of proteins that were modified by Woodward's reagent K. In addition, upon methylation of the carboxylates of a single protein, ovalbumin in this case, the degree of modification could be determined accurately and was confirmed by cation exchange chromatography elution profiles.

In the literature, much attention is paid to chromogenic assays to detect reactive groups on proteins. Such assays are valuable prerequisites for elucidating enzymatic mechanisms or determination of posttranslational processing of proteins. In addition, they are important tools in more applied research, for instance, for the determination of the extent of chemical modification of a protein or the degree of hydrolysis in protein hydrolysates.[1]

Several chromogenic reagents are available for the quantitative analysis of reactive amino acids on proteins. Commonly used methods are trinitrobenzene sulfonic acid (TNBS) and orthophthaldialdehyde (OPA) for the analysis of primary amino groups, such as in lysine residues and the N terminus.[2,3] For the quantification of free sulfhydryl groups, dithionitrobenzoic acid (DTNB, Ellman's reagent) can be used.[4] These assays are based on well-defined changes in the optical properties (absorbance or fluorescence) of a reagent upon reaction with the functional group of the amino acid and can be directly related to the number of reactive groups present. Moreover, these reagents are highly specific for certain residues and do not react with other residues in the protein.

For the determination of carboxylate groups, a method based on the reaction with 2-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K or WRK)[5,6] has been proposed by Sinha and Brewer.[7] This assay is based on the conversion of the reagent into a ketoketenimine around neutral pH, followed by either breakdown to the nonreactive ketoamide or reaction with organic carboxylates to form an enol ester.[8] Sinha and Brewer[7] found that the enol ester, resulting from the reaction of carboxylates present on protein with WRK in 75 mM Tris−HCl at pH 7.8, displays a peak absorbance at 340 nm with a molar extinction coefficient of 7000 M^{-1} cm^{-1}. Many researchers have determined the degree of modification of carboxylate groups by the use of WRK at 340 nm using this molar extinction coefficient.[9,10]

Llamas et al.,[11] however, reported that next to the carboxylate groups, also other nucleophiles in proteins could react with WRK. They showed that the enol ester of WRK formed with acetate displayed a maximum absorbance at 269 nm. Johnson and Dekker[12] and Bustos et al.[13] found that histidine and cysteine

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indeed reacted with WRK, as well, leading to an increased absorption at 340 nm.

The determination of the chemical functionality of biomaterials is essential not only for many industrial processes but also in life sciences insight into the availability of carboxylates in proteins is required to understand, for example, enzyme function. In view of the contradicting results found in the literature and the reported specificity for different substrates and measurement conditions, we reevaluated the above-described spectrophotometric method. The goal was to establish a reproducible and validated method for the quantification of carboxylates in proteins. First, the influence of reaction conditions was carefully studied. Next, the usefulness of the method for determining the amount of carboxylates present on a variety of proteins and on a single protein with different degrees of methylation of carboxylates is shown.

EXPERIMENTAL SECTION

Materials. Bovine milk β-lactoglobulin and hen egg white ovalbumin were purified to homogeneity as described previously.[14,15] Analytical-grade hen egg white lysozyme, bovine serum albumin (BSA), β-casein, κ-casein, trypsin, α-lactalbumin, 2-[*N*-morpholino]ethanesulfonic acid (MES) and 2-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K) were purchased from Sigma-Aldrich and used without further purification. All other chemicals used were of analytical grade and were used without further purification. Sephadex PD-10 gel filtration columns were obtained from Amersham-Pharmacia.

Spectrophotomeric Parameters. For all measurements, a Biospec UV-1601 from Shimadzu connected to a PC for data transfer was used. Cells were temperature-controlled by a CPS-240A cell positioner with an isothermic cell holder at 22 ± 0.1 °C. For recording absorbance spectra, quartz cells with a 1-cm path length were used. For protein concentration analysis by using the Biuret assay, plastic cells with 1-cm path length were used.

Woodward's Reagent K (WRK) Characteristics. *Stability of WRK Solution*. WRK was dissolved in 0.01 N HCl to a final concentration of 0.05 mM. Solutions were kept at 4 and 22 °C in the dark. Absorbance at 283 nm was measured at different time intervals of incubation at 22 °C.

Influence of pH on WRK Solution. The pH of 50 mL of 50 mM MES buffer containing 1% SDS and 1 M NaCl was adjusted to 2.0 with HCl. Subsequently, 100 μL of a 15.8 mM WRK solution in 0.01 N HCl was added to the buffer solution to make a final WRK concentration of 0.032 mM. After readjustment of the pH with NaOH and incubation at 22 °C for 15 min, 3 mL of this solution was transferred to a quartz cell, and the absorbance was measured directly at 283 nm. Absorbance data were corrected for the volume change caused by the addition of NaOH required to set the pH. The experiments were performed in triplicate.

Influence of Salt on the Stability of the WRK Solution. A range of 6 NaCl concentrations (10 mM to 1 M) were made by adding solid NaCl to 5 mL of 50 mM MES buffer containing 1% SDS (pH 5.0). To this solution, 10 μL of 15.6 mM WRK in 0.01 N HCl was added. Subsequently, it was incubated at 22 °C for 15 min. The absorbance at 283 nm was measured.

Kinetics of Spontaneous Degradation of WRK in Aqueous Solution. A 10-μL portion of a 15.6 mM WRK solution was added to 50 mM MES buffer containing 1% SDS at the appropriate pH (range between pH 4.0 and 6.0). Reactions were monitored at 283 nm and started within 20 s after mixing of the buffer with WRK at 22 °C. The absorbance measured is related to the initial absorbance of the sample at pH 6.0. Unreacted WRK was quantified at 283 nm using an extinction coefficient (ϵ) of 21 600 M^{-1} cm^{-1}.[11] First-order rate constants were determined from the logarithmic relationship between the absorbance as a function of time relative to the initial absorbance using a linear least-squares fit procedure. The experiments were performed at least in duplicate.

Protein Concentration. The protein concentration in samples was determined using the Biuret assay.[16,17] For the assay, solutions containing 0.3 and 10 mg protein/mL buffer were diluted to a final volume of 3 mL. Subsequently, 2 mL Biuret reagent was added. After mixing, the solutions were incubated for 30 min at room temperature. Absorbance was measured at 540 nm, and the reagent in absence of protein was used as reference. A calibration curve of BSA and an extinction coefficient of 0.0488 mg^{-1} cm^{-1} were used to evaluate the protein concentration. The protein concentration of ovalbumin was also measured spectrophotometrically using an extinction coefficient of 0.712 mL mg^{-1} cm^{-1} at 280 nm.[18] All determinations were performed in duplicate.

Methylation of Ovalbumin. Carboxylate groups in ovalbumin were methylated using an adapted version of the procedure described by Hoare and Koshland.[19] Four 100-mL batches of 10 mg/mL ovalbumin (0.22 mM) containing 1 M methylamine were prepared and brought to pH 4.6 with 0.02 M HCl. Subsequently, solid *N*-(3-dimethylaminoprpyl)-*N*′-ethylcarbodiimidehydrochloride (EDC from Fluka) was added stepwise to make final solutions of 10, 20, 50, and 100 mM EDC, while the pH was kept at 4.6 by the addition of 1 M NaOH using pH-stat equipment. When all added EDC was dissolved, the solutions were stirred for another 2 h. Next, the pH was raised to 8.0 by dropwise addition of NaOH (1 M) and the solution was stirred at room temperature for 24 h. To prevent microbial growth, 150 μg/mL sodium azide was added. After extensive dialysis (Visking, cutoff 14 000 Da) against demineralized water (4 °C), the mixtures were lyophilized and stored at −20 °C.

Cation-Exchange Chromatography. To demonstrate differences in net charge of the methylated ovalbumin variants, cation exchange chromatography (IEC) was performed. Separation of the different proteins was carried out on a Mono S column (Amersham-Pharmacia) with a bed volume of 1 mL supported by Äkta Purifier (Amersham-Pharmacia). The column was equilibrated with 50 mM sodium acetate (pH 5.0) at 20 °C. A 100-μL of a 5 mg/mL protein sample was applied to the column, and the flow rate was set to 0.5 mL/min. Proteins were eluted in 50 mM sodium acetate by applying a gradient of 1 M NaCl (pH 5.0) from

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0 to 100% over 20 column volumes. Protein detection took place at 220 nm.

Modification of Carboxylates by WRK. The available number of carboxylates on the protein was determined by a modified method, as originally described by Sinha and Brewer.[7]

Modification Reaction. Proteins were dissolved in 4.5 mL 50 mM MES buffer at pH 5.0 containing 1% SDS to a final concentration of 1 mM carboxylates. After addition of 0.5 mL of WRK from a 200 mM stock solution in 0.01 N HCl, the mixture was incubated for 15 min at 22 °C, while the pH was maintained at 5.0 ± 0.1 with 0.2 M NaOH using a pH-stat setup.

Removal of Unreacted WRK. The reacted protein was isolated from unbound WRK by applying the sample to a Sephadex PD-10 gel filtration column that was equilibrated with 50 mM MES (pH 5.0). A 2.5-mL sample was applied to the column, and subsequently, 3.5 mL of elution buffer (50 mM MES, pH 5.0) was put on top, as described by the manufacturer. The absorbance of the first fraction of 1.5 mL that was eluted from the PD-10 column was determined at 269 nm. The absorbance value was divided by the molar extinction coefficient from the calibration curve to calculate the molar concentration of carboxylates.

Calculation of the Number of Free Carboxylates per Molecule of Protein. First, the protein concentration in the reaction mix prior to the PD-10 column was determined by the Biuret assay. The Bradford assay and the measurement of the absorbance at 280 nm to determine the protein concentration were not applicable because of the presence of SDS and interfering absorbency at 269 nm from the WRK−carboxylate adduct, respectively. The protein concentration was multiplied by the dilution factor (1.9 ± 1.2%) of the PD-10 column to calculate the final protein concentration after the PD10 column. Molar weights of 42 750 (ovalbumin), 18 300 (β-lactoglobulin), 14 313 (lysozyme), 67 000 (BSA) 12 386 (cytochrome C), 23 583 (β-casein), 18 974 (κ-casein), 23 475 (trypsin), and 14 186 Da (α-lactalbumin) were used to analyze the data.

In this way, the number of free carboxylates was calculated by dividing the molar concentration of free carboxylates by the molar concentration of the protein. All protein samples were analyzed repeatedly, and the quantification appeared to vary within 5%.

RESULTS AND DISCUSSION

WRK (λ_{max} at 283 nm) is reported to be stable at pH values lower than 3, but it degrades spontaneously in aqueous solution into a ketoamide[6,8] (λ_{max} at 245 nm[11]) at higher pH values. Figure 1 shows the absorbance spectra of WRK in 0.01N HCl (dotted line). When the pH of the solution is adjusted to pH 12, the ketoamide is formed rapidly from WRK, resulting in a spectrum with a narrow band at 245 nm (dashed line). The small intensity at 283 nm might indicate that the spontaneous degradation was not completed yet. The solid line represents the reaction product of the protein and WRK. More specifically, at 269 nm, the carboxylate−WRK adduct had its maximum absorbance, while according to Johnsons and Dekker[12] and Bustos et al.,[13] at ∼340−350 nm, the cysteine−WRK and histidine−WRK adducts could be found.

Experimental Conditions. First, the stability of WRK in 0.01 N HCl (pH 2.0) was evaluated at 22 and 4 °C in the absence of light by recording the absorbance at 283 nm as a function of time.

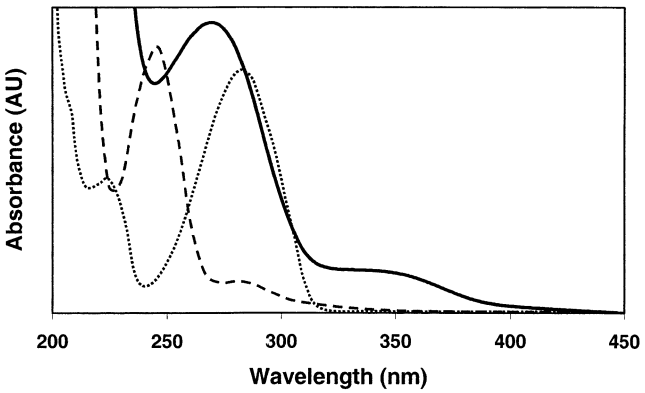

Figure 1. Absorbance spectra of WRK freshly dissolved in 0.01 N HCl (dotted line), ketoamide formed by spontaneous degradation of WRK upon prolonged incubation at pH 12 (dashed line), and ovalbumin−WRK adduct (solid line). For clarity reasons, the graphs have been rescaled to obtain graphs that are equal in height.

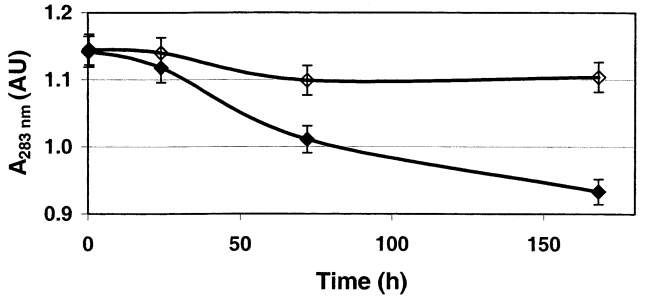

Figure 2. Absorbance at 283 nm of 0.05 mM WRK in 0.01N HCl stored at 4 °C (open symbols) and at 22 °C (closed symbols) as a function of time.

At both temperatures, no significant changes in intensity were observed within the first hour after dissolving WRK in 0.01 N HCl (Figure 2). Figure 2 also shows that at 4 °C, the absorbance at 283 nm decreased no more than 5% within 168 h. Upon incubation at 22 °C, however, the absorbance at 283 nm decreased almost 20%, illustrating the degradation of WRK. Storage under daylight enhanced the rate of degradation of WRK slightly (results not shown). Although WRK seems to be stable in 0.01 N HCl, it is advisable to store WRK stock solutions no longer than one week at 4 °C in the dark to avoid excessive degradation of WRK.

To examine the influence of pH on the spontaneous degradation of WRK into the ketoamide, MES buffer was set to pH 2.0 in the presence of 1 M NaCl to avoid major differences in ion strength. After dilution of the WRK stock solution into MES buffer, the absorbance at 283 nm (WRK) and 245 nm (ketoamide) were measured. This was repeated for all pH values in the same solution. All samples were incubated for 15 min. Figure 3A shows a gradual loss of absorbance between pH 4.0 and 5.5. Llamas et al.[11] already showed that the reaction rate of the spontaneous degradation of WRK into the ketoamide is pH-dependent and suggested a reaction scheme of two sequential first-order reactions, that is, first the formation of ketoketenimine from WRK and subsequently, the hydration of the ketoketenimine resulting in the ketoamide. Our experiments confirmed the initial appearance of a peak at 320 nm when the WRK solution was brought to pH 12, which disappeared again on a 30-min time scale coinciding with the appearance of a peak at 245 nm (not shown). Figure 3B shows the relation between the initial absorbance, determined at

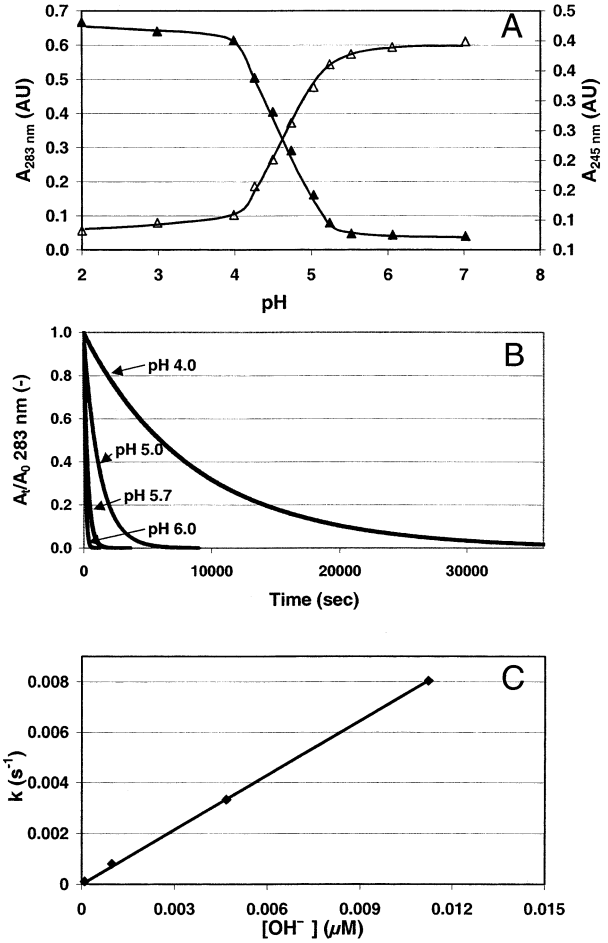

Figure 3. Kinetics of spontaneous degradation of WRK in 50 mM MES containing 1% SDS (pH 5.0) into ketoamide: (A) absorbance of WRK (closed symbol) and ketoamide (open symbol) in the same sample as a function of pH, (B) effect of pH on the decay of absorbance at 283 nm relative to the initial absorbance, and (C) kinetics of the disappearance of WRK as a function of concentration [OH^-].

pH 6.0, and the absorbance as a function of time at various pH values. The rate constant for each pH value was determined by a least-squares fitting procedure and is plotted in Figure 3C as a function of the concentration of hydroxyl ions. These rate constants are in agreement with values obtained by Llamas[11] and are related to the formation of the ketoketenimine. The rate constant is linearly dependent on the concentration base between pH 4.0 and pH 6.0. In all cases, 1 M NaCl was present in the sample to prevent significant differences in ionic strength related to the additions of acid and base to adjust the pH of the sample. When the effect of the ionic strength on the absorbance at 283 nm was tested by variation of the salt concentration between 0.01 and 1 M NaCl, no significant effect was observed (results not shown).

At pH 5.0, the reaction rate of spontaneous degradation of WRK is 8.11×10^{-4} s^{-1}. This indicates that one-half of the lifetime of WRK at pH 5 is ~14 min. The selection of a value of pH 5.0 to carry out all modification experiments is based on the dualism that on one hand, the WRK solution is more stable and available for reaction at low pH, whereas on the other hand, the protein carboxylates are more deprotonated (pK_a 3.5−4.5) at higher pH

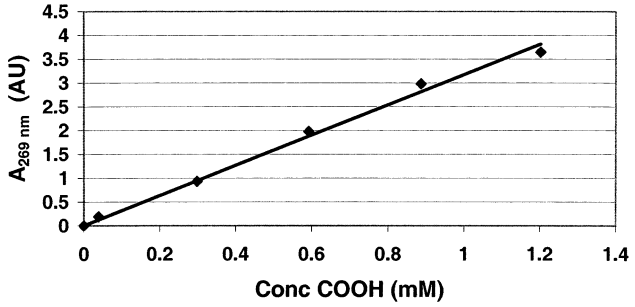

Figure 4. Calibration curve of the number of carboxylates present in ovalbumin in 50 mM MES containing 1% SDS (pH 5.0). Reaction took place in 15 min at room temperature with 20 mM WRK. Molar extinction coefficient = 3150 M^{-1} cm^{-1}.

and, therefore, more reactive. As described in the Experimental Section, a 15-min incubation time of the reaction mixture for the modification of protein with WRK was chosen. At this time interval, there will still be about 10 mM of the initial 20 mM WRK left for the modification of the 1 mM of carboxylate groups present in the incubation mixture.

The presence of any nucleophile in the sample, such as amino groups, causes the enol ester to be converted into the ketoamide, as described by Llamas et al.[11] Consequently, the presence of ammonium salts or compounds, such as urea, seriously hinder the analysis of the enol ester. In addition, the presence of the lysine side chains or the amino terminus of proteins is a potential threat for a reliable determination. Fortunately, the reaction rate of these nucleophiles with the enol ester is relatively slow, and only very high molar ratios of the nucleophile over enol esters give a significant underestimation of the total number of carboxylates on the protein. Nevertheless, it is important for a reliable analysis to keep a strict time schedule in the assay. Furthermore, other components containing carboxylates should be avoided to ensure a complete reaction of WRK with the protein carboxylates.

Molar Extinction Coefficient of the Enol Ester of Carboxylate−WRK Adduct. Complying with the above-described reaction conditions, the (molar) extinction coefficient of the enol ester of WRK reacted with protein carboxylates was determined from a calibration curve using ovalbumin (containing 48 carboxylates per protein). A range from 0.05 to 1.2 mM carboxylates was modified by 20 mM of WRK in MES buffer at pH 5.0. To denature the protein and, hence, make all carboxylates available for reaction, 1% SDS was added. A reference sample containing WRK and a sample of buffer containing ovalbumin were run in parallel, and the absorbance of the protein-free spectrum was subtracted from that of the sample containing protein. From the plot (Figure 4) of the absorbance at 269 nm as a function of the concentration of carboxylates in ovalbumin, a molar extinction coefficient of 3150 ± 100 M^{-1} cm^{-1} could be determined. Previously, Llamas[11] found for the enol ester upon reaction of WRK with acetate a molar extinction coefficient at 269 nm of 18 950 ± 500 M^{-1} cm^{-1}, which differs quite significantly from our value.

To confirm that all 48 carboxylate groups in ovalbumin were modified by WRK, MALDI-TOF experiments were performed, but not successfully. Working either in positive or negative mode of the MS setup did not gave any relative signal, while under these conditions, nonmodified protein gave excellent spectra. However, when a large variety of other proteins were tested for their

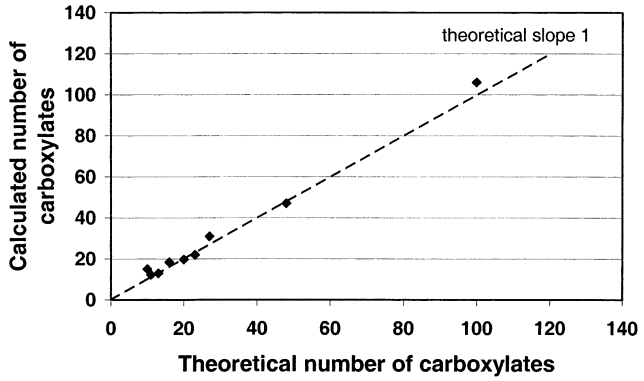

Figure 5. Scatter plot of the theoretical and found number of carboxylates. The dashed line with slope 1 displays the ideal relation between theoretical and found number of carboxylates of all proteins. The numbers of carboxylates of BSA (100 groups), β-lactoglobulin (27), lysozyme (10), cytochrome C (13), β-casein (22), κ-casein (16), trypsin (11), and α-lactalbumin (20) were calculated from the known primary sequence.

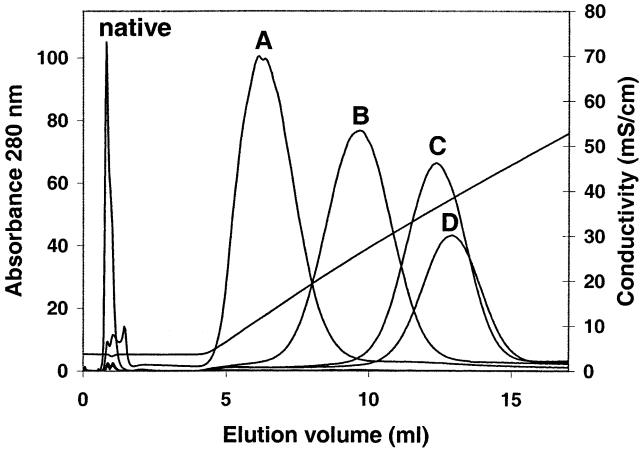

Figure 6. Typical chromatogram of cation exchange chromatography of native (0.06 mg) and various batches of methylated ovalbumin (0.5 mg) in 50 mM sodium acetate (pH 5.0). In addition, a gradient of sodium acetate containing 1 M NaCl is included. Methylated ovalbumin made by using a molar ratio COOH/EDC of 1:1 (A), 1:2 (B), 1:5 (C), and 1:10 (D).

carboxylate content, it was found that when this molar extinction coefficient was used, the total number of carboxylates per protein corresponded well to the theoretical number based on the primary sequence (Figure 5). The slope with value 1 gives the ideal relation between the theoretical number of carboxylates and the number found. Generally, a deviation of 5−10% between the theoretical and the determined number of reacted groups is found. Moreover, the figure shows that proteins with a wide range of molecular size can be analyzed.

Variation of the Number of Carboxylates in Methylated Ovalbumin. As an illustration of the application of this assay, the degree of modification of four methylated ovalbumin variants was determined. These variants were prepared by activation of carboxylates using different concentrations of EDC and subsequent methylation of carboxylates using the nucleophile methylamine. As a result, the four variants (denoted as variants A−D) differed in the average number of free carboxylates. Figure 6 shows the elution profiles of these ovalbumin variants applied to a cation exchange column. Native ovalbumin was eluted close to the void volume. The methylated variants were eluted successively according to their degree of modification presumed by the molar ratio of EDC added during the modification. The peaks of the variants were broader than the peak of native ovalbumin. This is most likely due to the heterogeneity of the samples in relation to the average number of modified carboxylates. The elution profile of variants C and D differed only slightly. Determination of the degree of modification of these variants by WRK confirmed the trend of methyl modification. Table 1 shows the values of the degree of modification. From these numbers, it is also clear that variant C and D do not differ very much (one carboxylate group).

Table 1. Number of Carboxyl Groups (COOH) in Native and Methylated Ovalbumin[a]

	mol total free COO^-/mol protein[b]	DM[c] (%)	theor net charge[d]
ovalbumin native	47 ± 2	0	−12
ovalbumin A	31 ± 2	35	+4
ovalbumin B	23 ± 2	51	+12
ovalbumin C	18 ± 2	62	+17
ovalbumin D	17 ± 2	64	+18

[a] Reactions were carried out in 50 mM MES containing 1% SDS (pH 5.0). Absorbance was measured at 269 nm. $\epsilon_{269} = 3150\ M^{-1}\ cm^{-1}$. [b] Average of three independent experiments. [c] Degree of modification. [d] Calculated from the moles of total free COO^-/mol of protein minus the theoretical number of positively charged residues (35).

CONCLUSIONS

In this work, a spectrophotometric assay for the determination of free carboxylates in proteins has been revisited. A number of critical reaction conditions were defined. Using a molar extinction coefficient of 3150 $M^{-1}\ cm^{-1}$ for the absorbance of the enol ester of WRK reacted with protein carboxylates, the total carboxylate content for a whole series of different proteins or proteins with different degrees of carboxylate modification could be determined accurately. Previous studies on the WRK reaction products focused either on the formation and stabilization of the ketoketenimine and the ketoamide or the enol ester with acetate (Llamas et al.[11]) or on the spectroscopic properties of WRK products at 340 nm (Sinha and Brewer[7]). The presentation of the enol ester of WRK reacted with carboxylates in proteins is new. This work, therefore, opens possibilities to better understand and control processing of biomaterials in general and, more specifically, in pharmaceuticals and the food or paper industries.

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